Color preferences and gastrointestinal-tract retention times of microplastics by freshwater and marine fishes

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(Citation)
Environmental Pollution,304:119253-119253

(Issue Date)
2022-07-01

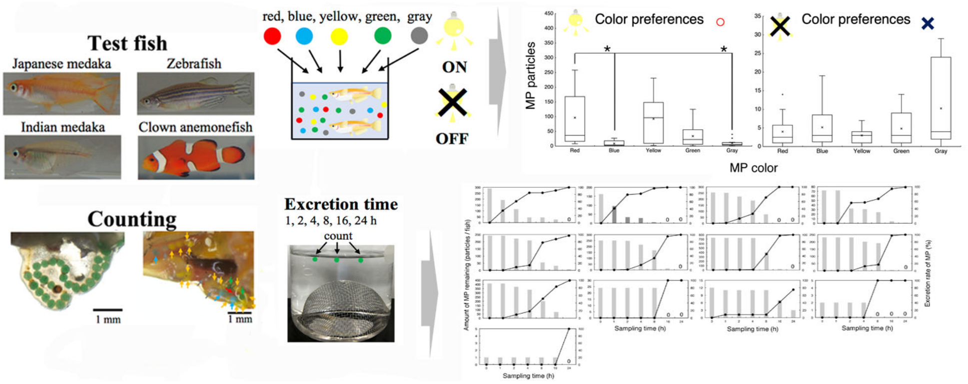
(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90009561

Highlights

- Clown anemonefish ingested the most MP particles, zebrafish ingested the second-most
- The most frequently ingested MP colors were red, yellow, and green in fish
- Clown anemonefish rely on color vision to recognize for certain MP colors
- MP excretion times varied widely among individuals of the same species


Test fish
Japanese medaka
Zebrafish
Indian medaka
Clown anemonefish
red, blue, yellow, green, gray
ON
OFF
Color preferences
MP particles
Red
Blue
Yellow
Green
Gray
MP color
Counting
1 mm
1 mm
Excretion time
1, 2, 4, 8, 16, 24 h
count
Amount of MP remaining (particles / fish)
Excretion rate of MP (%)
Sampling time (h)

Color preferences and gastrointestinal-tract retention times of microplastics by freshwater and marine fishes

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Abstract (196 words)

We examined ingestion and retention rates of microplastics (MPs) by two freshwater (Japanese medaka and zebrafish) and two marine fish species (Indian medaka and clown anemonefish) to determine their color preferences and gastrointestinal-tract retention times. In our ingestion experiments, clown anemonefish ingested the most MP particles, followed by zebrafish, and then Japanese and Indian medaka. Next, we investigated color preferences among five MP colors. Red, yellow, and green MP were ingested at higher rates than grey and blue MPs for all tested fish species. To test whether these differences truly reflect a recognition of and preference for certain colors based on color vision, we investigated the preferences of clown anemonefish for MP colors under light and dark conditions. Under dark conditions, ingestion of MP particles was reduced, and color preferences were not observed. Finally, we assessed gastrointestinal-tract retention times for all four fish species. Some individuals retained MP particles in their gastrointestinal tracts for over 24h after ingestion. Our results show that fish rely on color vision to recognize and express preferences for certain MP colors. In addition, MP excretion times varied widely among individuals. Our results provide new insights into accidental MP ingestion by fishes.

Keywords: **color preferences, microplastic, ingestion, fish**

Introduction

In recent years, microplastic (MP) pollution (i.e., pollution involving plastic particles of diameter < 5 mm) has become a serious environmental problem around the world (reviewed by Bagaev et al., 2021; Galarpe et al., 2021; Li et al., 2016). Pinherio et al. (2021) reviewed MP concentrations in marine and freshwater environments in various countries including the United Kingdom, United States, Brazil, China, France, Germany, India, Italy, Australia, Indonesia, and Japan. Furthermore, MP contamination has been repeatedly identified in marine (reviewed by Hidalgo-Ruz et al., 2012) and freshwater (reviewed by Yang et al., 2021) environments worldwide. Therefore, the ecotoxicity of MP in aquatic organisms has become a topic of increasing concern (Galloway and Lewis, 2016).

To date, various field and laboratory studies have examined the influence of MP on fishes. In the laboratory, MP ingestion and uptake into the gastrointestinal tract has been observed in goldfish (*Carassius auratus*) (Grigorakis et al., 2017), zebrafish (*Danio rerio*) (Kim et al., 2019), mummichog (*Fundulus heteroclitus*) (Ohkubo et al., 2020), and red seabream (*Pagrus major*) (Ohkubo et al., 2020). In addition, MP has been identified in wild fish captured in the Lijiang River in China (Zhang et al., 2021), the Mondego Estuary in Portugal (Bessa et al., 2018), the Xingu River in Brazil (Andrade et al., 2019), the Pacific coast of Ecuador (Alfaro-Núñez et al., 2021), and the northern Bay of Bengal in Bangladesh (Hossain et al., 2020). These results indicate that MP ingestion by fishes is widespread.

Various colors of MP (e.g., transparent, black, blue, grey, green, red, purple, and yellow) are present in marine (Courtene-Jones et al., 2017; Dai et al., 2018; Lusher et al., 2014; Martin et al., 2017) and freshwater environments (Li et al., 2018; McNeish et al., 2018; Su et al., 2016; Wu et al., 2020). Colored MP has also been detected in wild fish (Bessa et al., 2018; Jawad et al., 2021; Kazour et al., 2020), suggesting that colored MP can potentially be mistaken for natural prey. If various marine and freshwater fishes exhibit color preferences for MP ingestion, this would be important for understanding the harmful influence of MP on wild fishes.

Another important factor for estimating the harmful influence of MP on fishes is MP retention and excretion times. This information is needed to assess the extent to which hazardous chemical substances adsorbed onto MPs can be absorbed into the body after ingestion. However, to our knowledge, there are only a few laboratory reports that currently provide this data (i.e., for goldfish [Grigorakis et al., 2017], mummichog [Ohkubo et al., 2020], and red seabream [Ohkubo et al., 2020]). More data are needed on retention and excretion times in various fishes including both freshwater and marine species, because MP is detected in both freshwater and marine environments.

In our study, we examine two freshwater fish species (Japanese medaka *Oryzias latipes* and zebrafish *Danio rerio*) and two marine species (Indian medaka

Oryzias melastigma and clown anemonefish *Amphiprion ocellaris*). Japanese medaka typically inhabits gently flowing rivers and waterways in Japan (Sakaizumi 1986; Sakaizumi and Jeon 1987), and zebrafish inhabits moderately flowing to stagnant clear waters in India, Pakistan, Bangladesh, Nepal, and Bhutan (Lawrence, 2007). Japanese medaka and zebrafish are widely used as model freshwater fishes for ecotoxicological studies under OECD test guidelines (OECD, 2012, 2013, 2019). Indian medaka inhabits coastal marine waters and freshwater bodies in Pakistan, India, Myanmar, and Thailand (Dong et al., 2014). Recently, Indian medaka has also become a common choice as a model marine fish for ecotoxicological studies (He et al., 2021; Horie et al., 2019; Wang et al., 2020; Zheng et al., 2020). Clownfish is found throughout the Indo-Pacific (Camp et al., 2016; Chen and Hsieh., 2017; Chen et al., 2018).

First, we identify any preferences for MP color among Japanese medaka, zebrafish, Indian medaka, and clown anemonefish under laboratory conditions. Next, we estimate MP retention times for all four species. The findings of this study will be useful for further estimation of MP ecotoxicity in these species.

2. Materials and methods

All animal experiments were conducted according to the relevant national guidelines (Act on Welfare and Management of Animals, Ministry of the Environment, Japan) and the fish used in the present study were handled according to the animal care and use guidelines of Kobe University. All animal experiments were approved by the institutional animal care and use committee, Research Center for Inland Sea, Kobe University (Permission number, 2021-04). Our research was also performed in accordance with the ARRIVE guidelines.

2.1. Study organisms and MPs

We examined two freshwater fishes, Japanese medaka and zebrafish, and two marine fishes, Indian medaka and clown anemonefish. Japanese medaka, zebrafish, Indian medaka, and Clown anemonefish were bred at Kobe University (Hyogo, Japan). Both marine and freshwater fishes were maintained at a water temperature of 25 ± 2 °C (mean ± SD) by using a recirculating system. Marine fishes were maintained at a salinity of 32 PSU. Fish were raised under an artificial photoperiod of 12-h/12-h light/dark. Marine species were kept in artificial seawater (Marine ART Hi; Osaka Yakken Co. Ltd, Osaka, Japan). In this study, we used young fish and genetic sex was unknown. Average total body lengths (mm) and wet body weights (mg) of each species were as follows: 15.1 ± 0.1 mm and 30.0 ± 0.8 mg for Japanese medaka (age: 1-2 months after hatching); 12.6 ± 0.1 mm and 12.2 ± 0.4 mg for zebrafish (age: 1-2 months after hatching); 16.7 ± 0.1 mm and 40.6 ± 0.8 mg for Indian medaka (age: 1-2 months after hatching); and 31.1 ± 0.2 mm and 484.7 ± 11.9 mg for clown anemonefish (age: 4-6 months after hatching).

Polyethylene microspheres were purchased from Cospheric (Santa Barbara, CA, USA) in five colors (red, blue, yellow, green, and gray). The average diameter and particle density of each color was as follows. Red: 219.2 ± 22.6 µm, 0.98 g/cc; blue: 279.0 ± 17.0 µm, 1.00 g/cc; yellow: 256.9 ± 21.2 µm, 1.00 g/cc; green: 253.6 ± 20.4 µm, 0.98 g/cc; gray: 257.7 ± 21.7 µm, 1.00 g/cc (Supplementary Figure 1).

2.2. MP ingestion assays (Experiments 1 and 2)

A flow chart summarizing the experimental procedure for all experiments (Experiments 1–4) is shown in Figure 1. For MP ingestion, we conducted two experiments: in Experiment 1, we exposed each of the four fish species to a single color (red, blue, yellow, green, or gray) of MP; in Experiment 2, we exposed each species to a mix of the five MP colors to test for the presence of a color preference. Seven fish were placed in 5-L glass tanks filled with 4 L of water, and two replicate tanks were used for each exposure (i.e., *n* = 14 per exposure).

Before establishing the nominal MP concentrations for exposures, we assessed the maximum MP ingestion amount for each fish species. The maximum MP ingestion test lasted for 4 h (until feeding behavior was no longer observed for four fish species). The Japanese medaka, zebrafish, and Indian medaka individuals that ingested the most MP particles in our assessment each ingested fewer than 100 particles, and the clown anemonefish that ingested the most MP particles ingested fewer than 1000 particles

(Figure 2). The weight of 1000 MP particles is approximately 10 mg. Because each 5-L glass tank contained 4 L of water and seven fish in our exposure experiments, the nominal MP concentration for Experiment 1 was set at 1.75 mg/L for Japanese medaka, zebrafish, and Indian medaka, and 17.5 mg/L for clown anemonefish. For Experiment 2, the concentration for each MP color was set at 1.75 mg/L (i.e., a total MP concentration of 8.75 mg/L) for each of the three fish species. In case of clown anemonefish, the concentration for each MP color was set at 17.5 mg/L (i.e., a total MP concentration of 87.5 mg/L).

Fish were placed into 5-L glass tanks 24 h prior to the start of each exposure experiment, and were not fed during this time to ensure that their gastrointestinal tracts would be empty. Each MP exposure test lasted for 4 h (until feeding behavior was no longer observed for four fish species). After 4 h of exposure, fish were anesthetized (MS-222 at a concentration of 200 mg/L) and their gastrointestinal tracts dissected, and the number of MP particles in each gastrointestinal tract was counted under a stereomicroscope (SZ61, Olympus).

2.3. MP color-preference assays (Experiments 3)

In Experiment 3, we examined clown anemonefish (which ingested the most MP particles in pretests prior to Experiment 1) to clarify whether fish rely on their color vision to recognize MP colors. Exposure conditions were similar to those in Experiment

2. Seven fish were placed in 5-L glass tanks, two replicate tanks were used for each exposure (i.e., *n* = 14 per condition), and the exposure concentration for each MP color was set to 17.5 mg/L (i.e., total MP concentration of 87.5 mg/L).

Ten days prior to each exposure experiment, in order to reset the circadian rhythm of the study animals, the husbandry schedules for the fish were reversed; i.e., feeding was performed in the dark, and water changes were performed in the light. Fish were then placed into 5-L glass tanks 24 h prior to the start of each exposure experiment, and were not fed during this time to ensure that their gastrointestinal tracts would be empty. Next, MP exposure tests were carried out for 4 h (until feeding behavior was no longer observed for clown anemonefish) under dark conditions. After the 4-h exposure, fish were anesthetized (MS-222 at a concentration of 200 mg/L) and their gastrointestinal tracts dissected, and the number of MP particles in each gastrointestinal tract was counted under a stereomicroscope (SZ61, Olympus).

2.4. MP retention in and excretion from the gastrointestinal tract (Experiments 4)

In Experiment 4, we examined the gastrointestinal tract retention and excretion times of green MP (which was the most commonly ingested MP color in Experiment 1) in each fish species. Exposure conditions were the same as in Experiment 1. After 4 h of exposure, each fish was transferred from its 5-L glass exposure tank to a 1-L glass beaker with a stainless-steel screen at the bottom for the excretion experiment.

The fish were confined under the stainless-steel screen (mesh size: 2 mm (width)*2 mm (height)) (to prevent re-feeding for the excretion MP) so that the MP, once excreted, could swim freely to the surface due to their low density. Once at the surface, they were collected by using glass pipett and were counted under a stereomicroscope in directly (SZ61, Olympus). A total of 14 glass beakers were prepared to accommodate the 14 experimental fish. After 1, 2, 4, 8, 16, and 24 h, the number of MP particles excreted from each fish was counted, and all MP were removed from the 1-L beaker. After the excretion test, fish were anesthetized (MS-222 at a concentration of 200 mg/L) and their gastrointestinal tracts dissected, and the number of MP particles in each gastrointestinal tract was counted under a stereomicroscope (SZ61, Olympus).

2.5. Statistical analysis

All data were analyzed in Microsoft Excel. To analyze significant differences between light and dark conditions, we used open source statistical software R (http://www.R-project.org/) and the package *Rcmdr* (Fox and Bouchet-Valat 2018) to test for homogeneity of variance using Bartlett’s test (significance level, 5%), and tested for significant differences using Steel’s test. To analyze color preference in clown anemonefish, we used Steel–Dwass multiple-comparison tests (significance level, 5%).

3. Results

3.1. MP uptake by the four fish species

Table 1 shows a summary of the number of fish of each species in Experiment 1 that ingested MP of each color. Zebrafish and clown anemonefish readily ingested MP of all colors, but Japanese medaka and Indian medaka mainly ingested green MP. The proportion of individuals that ingested MP of any color was highest for clown anemonefish, followed by zebrafish, Japanese medaka, and Indian medaka.

Figure 2 shows the number of MP particles ingested by the four fish species. In Japanese medaka, almost all individuals ingested fewer than 10 MP particles, with the exception of one individual that ingested 31 blue MP particles and three individuals that ingested 28, 29, and 77 green MP particles, respectively (Fig. 2a). In zebrafish, almost all individuals ingested between 10 and 30 MP particles, and the most MP particles were ingested by an individual that consumed 63 yellow MP particles (Fig. 2b). In Indian medaka, almost all individuals ingested fewer than 10 MP particles, with the exception of two individuals that ingested 12 and 24 red MP particles, respectively and one individual that ingested 15 green MP particles (Fig. 2c). Clown anemonefish ingested the most MP in total. Almost all individuals ingested more than 100 MP particles, including one individual that ingested 744 red MP particles (Fig. 2d). There were no consistent differences in MP intake by color across the four species.

3.2. Color preferences for MP intake

The number of fish that ingested MP in Experiment 2 was as follows: Japanese medaka, 4 of 14; zebrafish, 6 of 14; Indian medaka, 5 of 14; and clown anemonefish, 12 of 14 (Fig. 3). Figure 3 shows color preferences for MP ingestion in Japanese medaka (Fig. 3a), zebrafish (Fig. 3b), and Indian medaka (Fig. 3c); the results for clown anemonefish are shown separately (Fig. 4b). Overall, the MP colors that were most frequently ingested by Japanese medaka, zebrafish, Indian medaka, and clown anemonefish were red, yellow, and green, and the least frequently ingested colors were blue and gray, although significant differences could not analyze due to the number of MP particles ingested were low level.

Next, we examined the presence or absence of color preferences (Fig. 4; Experiment 2 and 3). In this experiment, we used clown anemonefish, which ingested the most MP particles of the species examined in Experiment 1. Clown anemonefish ingested fewer MP particles under dark conditions than under light conditions (Fig. 4a). Moreover, whereas red, yellow, and green MP were preferred under light conditions (Fig. 4b), no color preference was apparent under dark conditions (Fig. 4b). Results of steel–Dwass multiple-comparison tests (significance level, 5%), showed that red MPs was significantly more ingested in comparison to blue and gray MP under light conditions (Fig. 4c), on the other hand, under dark conditions, no significant differences were observed (Fig. 4c).

3.3. Time-course of MP retention in and excretion from the gastrointestinal tract

The results of the MP retention and excretion tests were as follows. Eight of 14 Japanese medaka (57%) excreted all of the MP contained in their gastrointestinal tracts within 24 h (Figure 5); some residual MP remained in the gastrointestinal tracts after 24 h in the remaining six fish. Nine of 14 zebrafish (64%) excreted all of the MP contained in their gastrointestinal tracts within 24 h (Figure 6); only one individual excreted less than 90% of its ingested MP within the 24-h observation period. All 14 Indian medaka excreted all of the MP contained in their gastrointestinal tracts within 24 h (Figure 7). Nine of 13 clown anemonefish (69%) excreted all of the MP contained in their gastrointestinal tracts within 24 h (Figure 8); only one individual excreted less than 20% of its ingested MP within the 24-h observation period.

There was no consistent pattern of MP excretion among fish species, and the excretion time varied widely among individuals of the same species. In addition, there was no apparent difference in MP excretion time caused by the presence (clown anemonefish) or absence (Japanese medaka, zebrafish, and Indian medaka) of a stomach.

4. Discussion

This study is the first to evaluate whether fish differentiate among five distinct colors of MP under laboratory conditions and can preferentially ingest MP on this basis.

Our results on MP ingestion highlight the magnitude of variability in MP ingestion among species and individuals. Recently, Ohkubo et al (2020) reported that the number of MP particles (diameter, 250–300 µm; color, yellow) identified in the gastrointestinal tracts of mummichog was 352 ± 240 at an exposure concentration of 3 mg/L, and the number identified in red seabream was 41.8 ± 14.9 at an exposure concentration of 0.9 mg/L. In the present study, the average number of MP particles of the type examined in the above reports (diameter, 250–300 µm; color, yellow) identified in gastrointestinal tracts was 0, 25 ± 18, and 1 in Japanese medaka, zebrafish, and Indian medaka, respectively, at an exposure concentration of 1.75 mg/L; in clown anemonefish the number was 215 ± 198 at an exposure concentration of 17.5 mg/L. Some of the species (Japanese medaka, zebrafish, and Indian medaka) do not possess stomachs, and clown anemonefish do. However, the number of ingested MP particles does not appear to depend strongly on the presence or absence of a stomach. Further studies on other fish species could help identify commonalities between fish that are or are not prone to accidental MP ingestion.

Our results on color preferences contrast with reports from the field. To date, there have been numerous reports of plastics in the gastrointestinal tracts of wild fishes. For example, blue and black MP filaments and blue, green, and black MP fragments were frequently identified in Atlantic horse mackerel (*Trachurus trachurus*) from the central Mediterranean Sea (Chenet et al., 2021). Along the southwest coast of the United

Kingdom, grey/transparent MP were the most frequently ingested by small-spotted catshark (*Scyliorhinus canicula*), and green was the least frequently ingested (Morgan et al., 2021). In the Adriatic Sea in Italy, black, tan, and blue MP fragments were frequently found in the stomachs of *Sardina pilchardus*, and black and blue MP fragments were frequently identified in the stomachs of *Engraulis encrasicolus* (Renzi et al., 2019). By contrast, in our laboratory study, red, yellow, and green MP particles were frequently observed in the gastrointestinal tracts of zebrafish, Indian medaka, and clown anemonefish, and blue and gray particles were less common. This may indicate that color preferences differ between field and laboratory conditions. Future studies on the same species in the field and laboratory will clarify whether this is the case.

In general, vertebrates possess two types of photoreceptor cells: cones and rods. Cone cells enable color vision during light conditions, and rod cells, which do not distinguish among colors, are more sensitive under low-light conditions, meaning that they are used most heavily in the dark. Bony fishes such as goldfish and zebrafish have four spectral cone types comprising alternating rows of double cones with red (LWS) and green (RH2) members and single blue (SWS2) and UV (SWS1) cones (Allison et al., 2010; Baden and Osorio, 2019; Engström, 1960; Raymond et al., 1993). On the other hand, the few shark species that have been studied only possess a single spectral class of cone with LWS (Hart et al., 2011; Hart et al., 2020; Theiss et al., 2012), indicating that they are almost certainly cone monochromats and do not possess color vision (Schluessel et al., 2014). Recently, Mitchell et al (2021) reported that clown anemonefish has four spectral cone types including LWS, RH2, SWS2, and SWS1, and rhodopsin 1 (rod opsin, RH1). Therefore, this report and our results indicate that clown anemonefish are able to recognize MP colors. Under light conditions, the species showed a preference for red, yellow, and green MP, but under dark conditions, the number of MP particles ingested declined and color preferences were not observed. Various colors of MP have been identified in the field (Chenet et al., 2021; Morgan et al., 2021; Renzi et al., 2019), and our study identified color preferences for MP ingestion in clown anemonefish (a marine species). Further studies are needed to determine the presence or absence of color preferences in other fish species to further our understanding of MP ingestion in the field. In addition, color preference data could help clarify whether fish are misidentifying MP as food.

To our knowledge, reports on MP retention and excretion times in fish in the laboratory remain scarce, although there have been many reports of MP being identified in the gastrointestinal tracts of wild fishes (Bessa et al., 2018; Jawad et al., 2021; Kazour et al., 2020). Excretion times observed in the present study can be divided into three patterns: (1) immediate excretion, in which most MP is excreted within the first 4 h; (2) gradual excretion, in which MP is excreted gradually over 24 h; and (3) delayed excretion, in which most MP is excreted after 16 h. A previous study (Ohkubo et al., 2020) on mummichog and red seabream also identified the above three patterns. In the future, it will be necessary to investigate whether these three patterns apply to other fish species. In addition, why were there observed the difference of excretion patterns in same fish species? Various factors such as gender differences, health conditions, or fitness differences, etc. can be considered, therefore, further research is needed in the near future.

In contrast to previous reports, our results show that MP can remain in fish gastrointestinal tracts for a prolonged period following ingestion. Ohkubo et al (2020) reported that over 95% of ingested MP was excreted within 25 h in mummichog and red seabream. Similarly, Naidoo and Glassom (2019) reported that only a few particles of negligible mass remained 24 h after MP exposure in *Ambassis dussumieri*. By comparison, we found that some Japanese medaka, zebrafish, and clown anemonefish retained MP in the gastrointestinal tract even 24 h after MP ingestion. This indicates that the time required for MP excretion does not depend on fish species, but on the individual. In the future, we intend to examine excretion rates across a longer time horizon to clarify the time required to discharge all ingested MP particles.

5. Conclusion

To assess the impact of MP on fish, it is essential to first understand accidental MP ingestion and gastrointestinal-tract retention times. Ours is the first study to identify color preferences among teleost fishes for MP ingestion, and also provides valuable data on MP excretion times for both freshwater and marine fishes. The freshwater zebrafish and marine clown anemonefish both ingested large numbers of MP particles compared to Japanese and Indian medaka. Also, red, yellow, and green MP was more frequently ingested than grey and blue MP overall. The number of MP particles ingested was reduced, and color preferences were not observed under dark conditions in clown anemonefish. These findings suggest that clown anemonefish rely on color vision to recognize MP colors and express color preferences. MP excretion times varied widely among fish of the same species, and some individuals still had MP particles remaining in their gastrointestinal tracts more than 24 h after exposure. These findings provide new insights into accidental MP ingestion by fishes.

Acknowledgments

The authors thank Professor. Taisen Iguchi, Yokohama City University, Japan, for his critical readings of this manuscript. This study was supported in part by the Kurita Water and Environment Foundation (20B021) to Y.H, and by the New Energy and Industrial Technology Development Organization (JPNP18016) to H.O.

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Figures

Figure 1. Experimental flow chart. In Experiment 1, fish from each of the four species were each exposed to a single color (red, blue, yellow, green, or gray) of microplastic (MP). In Experiment 2, fish were exposed to a mix of the five MP colors under light conditions. In Experiment 3, clown anemonefish were exposed to a mix of the five MP colors under dark conditions. In Experiment 4, we determined gastrointestinal tract retention and excretion times in each of the four fish species using green MP.

Figure 2. The number of microplastic (MP) particles in the gastrointestinal tracts of (**a**) Japanese medaka, (**b**) zebrafish, (**c**) Indian medaka, and (**d**) clown anemonefish after exposure to various MP colors. Circles show values for each individual.

Figure 3. The number of microplastic (MP) particles of various colors observed in the gastrointestinal tracts of (**a**) Japanese medaka, (**b)** zebrafish, and (**c**) Indian medaka after exposure to a mix of five MP colors. *X*-axis values show ID numbers for each fish: i.e., exposure tests were conducted on a total of four Japanese medaka, six zebrafish, and five Indian medaka.

Figure 4. Color preferences of clown anemonefish under light and dark conditions. The number of fish that ingested MP was 12 of 14 in both light and dark conditions. (**a**) The

number of microplastic (MP) particles observed in clown anemonefish gastrointestinal tracts under light (circles) and dark (triangles) conditions. (**b**) The number of MP particles of various colors observed in the gastrointestinal tracts of clown anemonefish under light conditions and dark conditions after exposure to a mix of five MP colors. *X*-axis values show ID numbers for each fish: i.e., exposure tests were conducted on a total of twelve individuals under light and dark conditions. (**c**) Box plot of the number of MP particles of various colors observed in the gastrointestinal tracts of clown anemonefish. *Values that are significantly different (Steel's test or Steel–Dwass multiple-comparison tests; $*P < 0.05$)

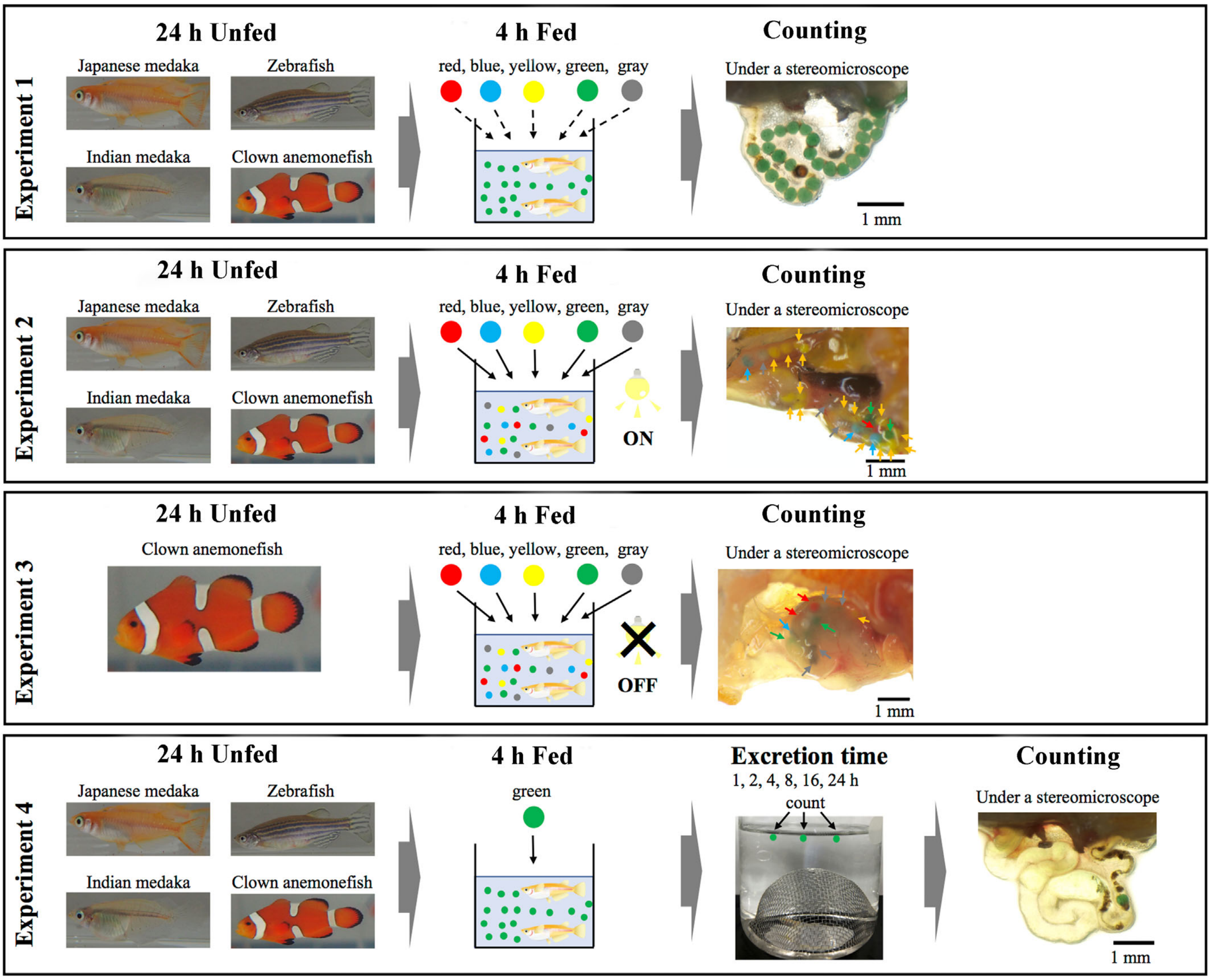
Figure 5. Microplastic (MP) excretion in Japanese medaka. Bars show numbers of ingested MP particles remaining in fish gastrointestinal tracts at each sampling time, and lines show % of total excreted MPs at each sampling time.

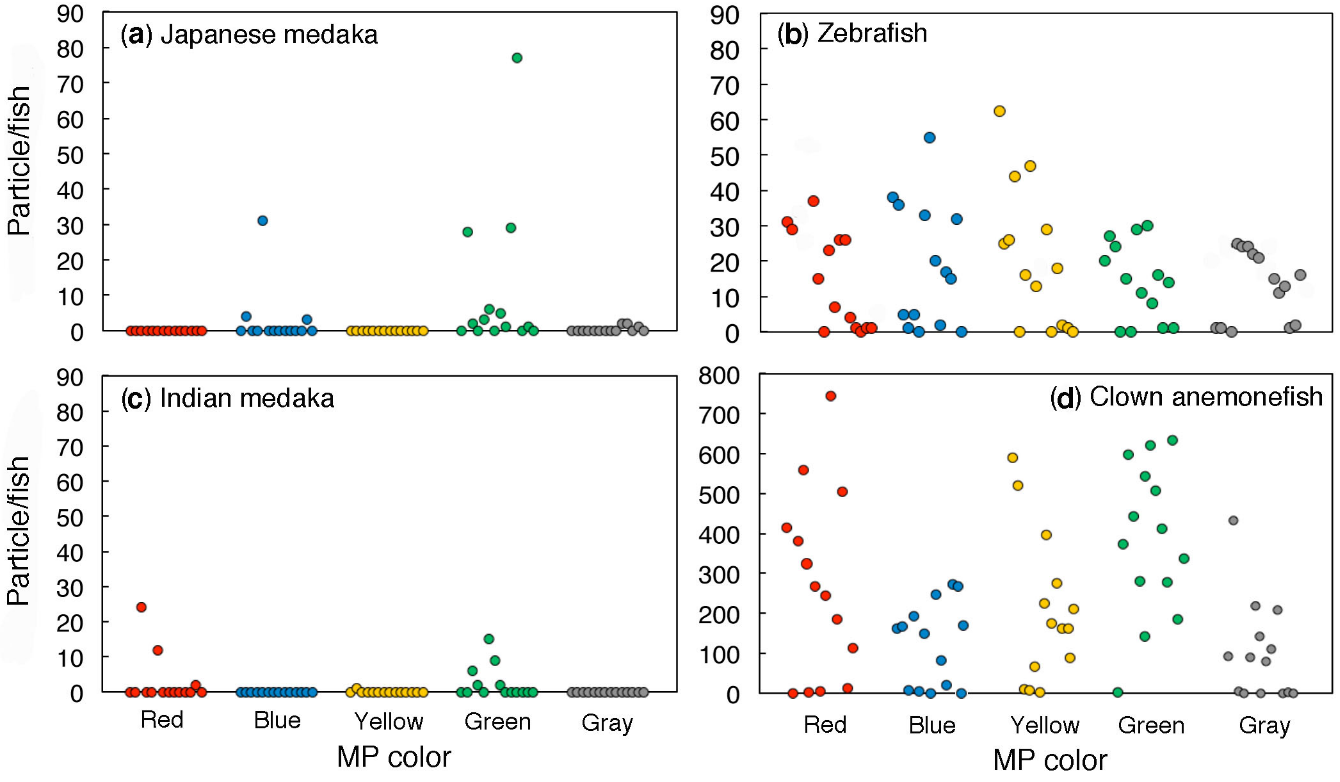
Figure 6. Microplastic (MP) excretion in zebrafish. Bars show numbers of ingested MP particles remaining in fish gastrointestinal tracts at each sampling time, and lines show % of total excreted MPs at each sampling time.

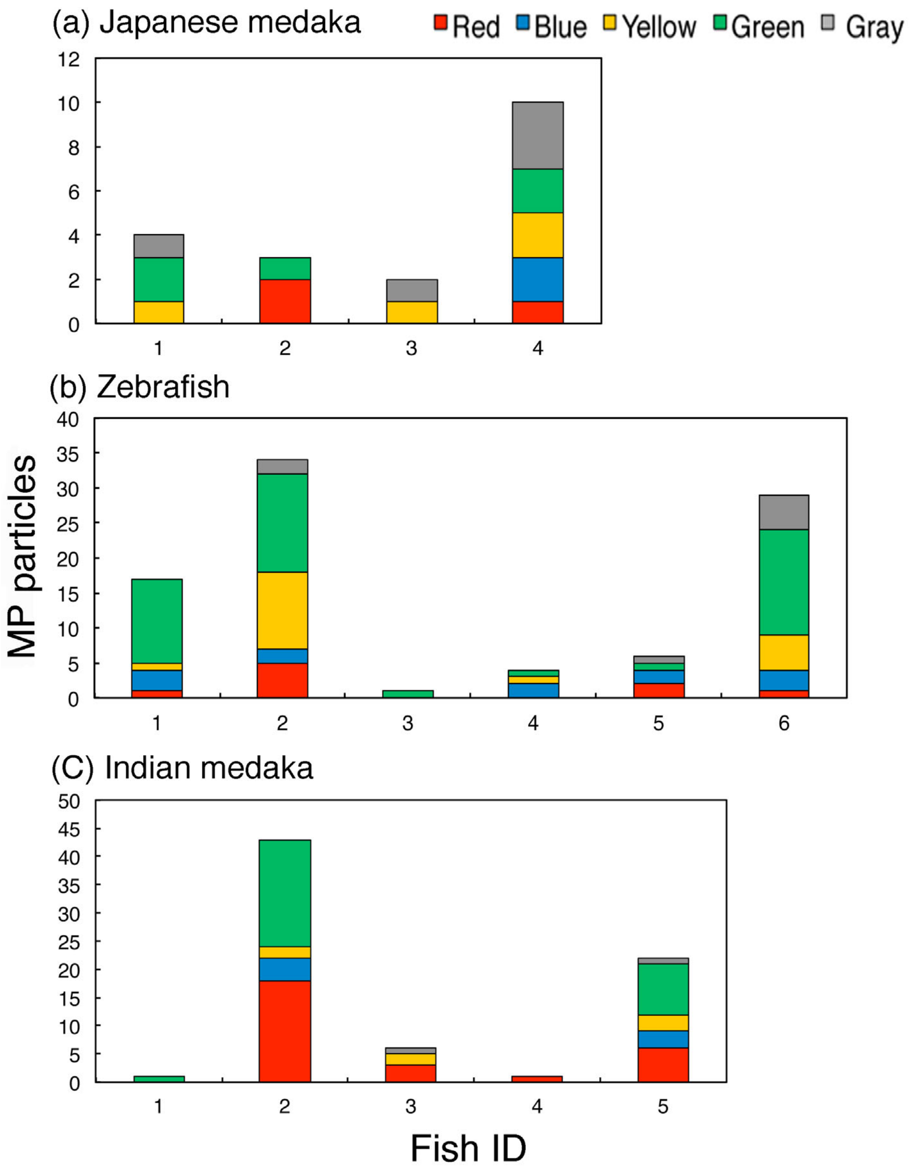
Figure 7. Microplastic (MP) excretion in Indian medaka. Bars show numbers of ingested MP particles remaining in fish gastrointestinal tracts at each sampling time, and lines show % of total excreted MPs at each sampling time.

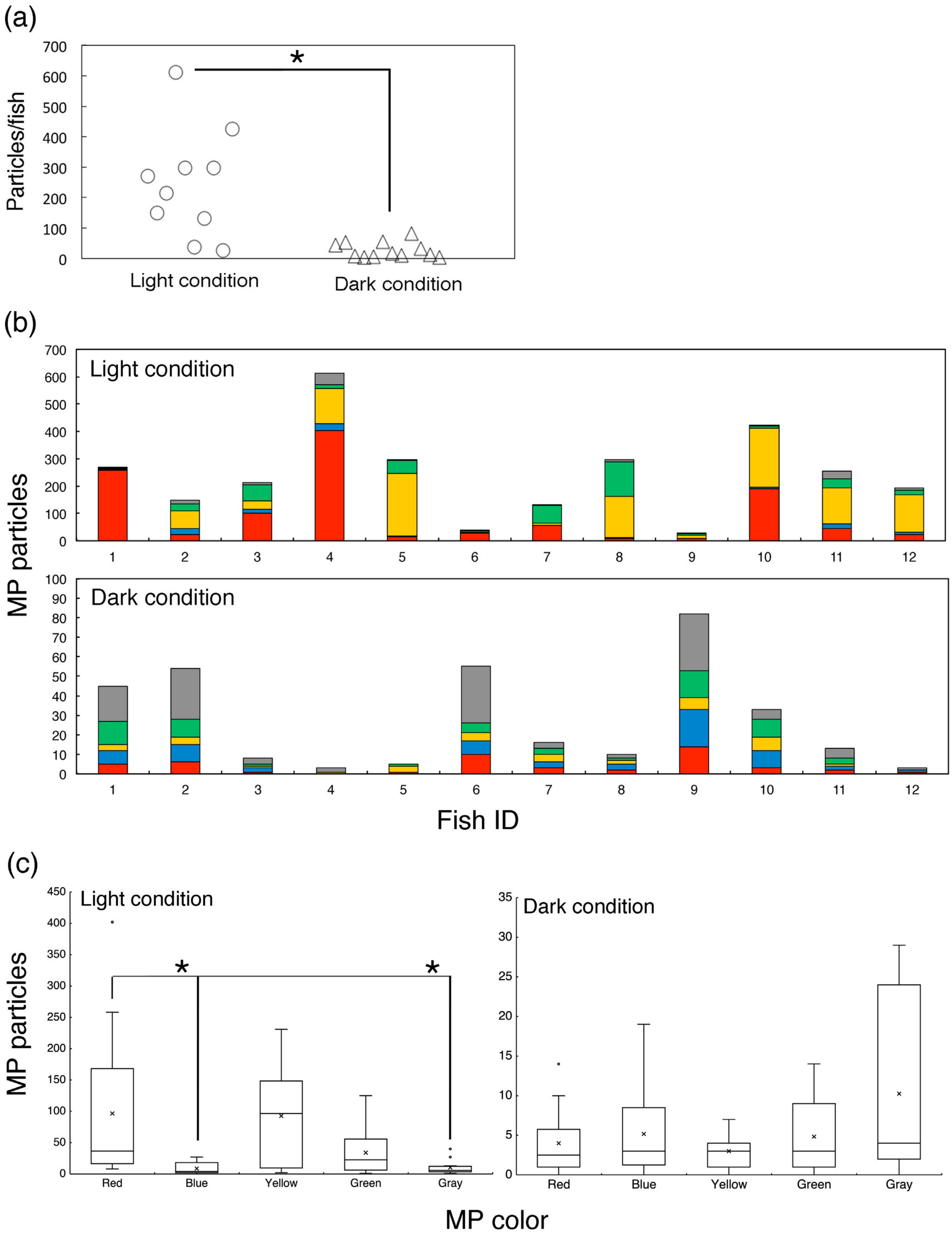
Figure 8. Microplastic (MP) excretion in clown anemonefish. Bars show numbers of ingested MP particles remaining in fish gastrointestinal tracts at each sampling time, and lines show % of total excreted MPs at each sampling time.

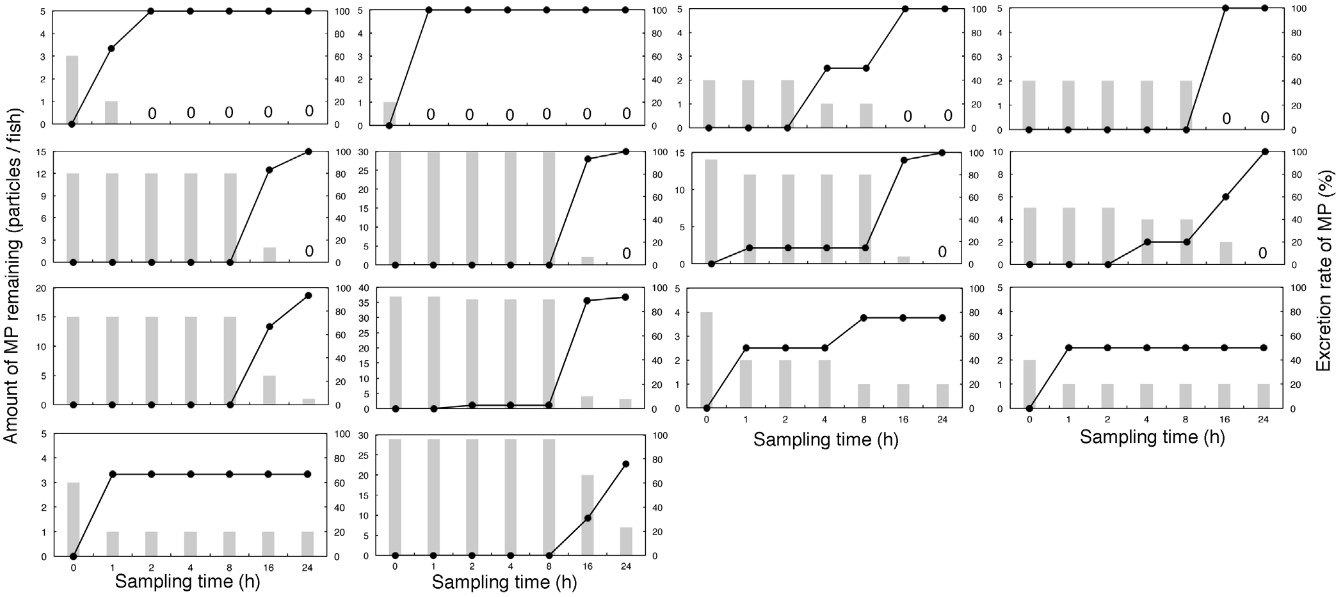
Supplementary Figure 1. Photographs of polyethylene microspheres in the five colors used in the study: (**a**) red, (**b**) blue, (**c**) yellow, (**d**) green, and (**e**) gray. Average particle diameters for each color (**f**) were measured by using a stereomicroscope (SZ61, Olympus). Error bars show ± standard deviation (*n* = 100 per color).

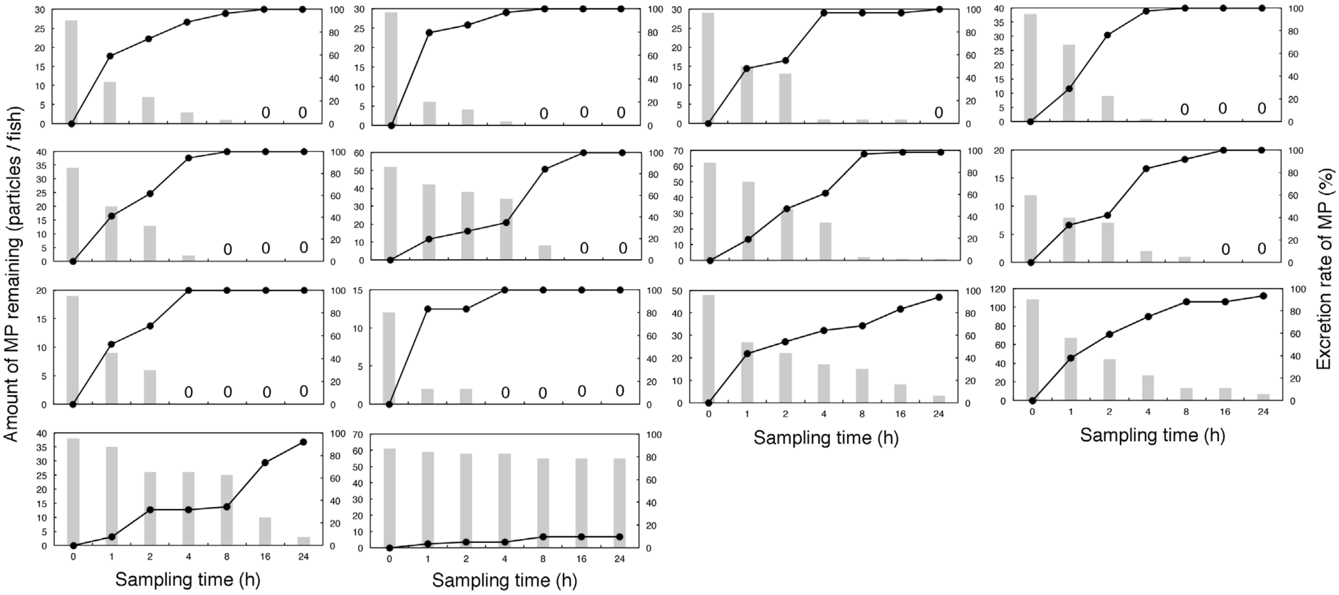

Experiment 1
24 h Unfed
Japanese medaka
Zebrafish
Indian medaka
Clown anemonefish
4 h Fed
red, blue, yellow, green, gray
Counting
Under a stereomicroscope
1 mm
Experiment 2
24 h Unfed
Japanese medaka
Zebrafish
Indian medaka
Clown anemonefish
4 h Fed
red, blue, yellow, green, gray
ON
Counting
Under a stereomicroscope
1 mm
Experiment 3
24 h Unfed
Clown anemonefish
4 h Fed
red, blue, yellow, green, gray
OFF
Counting
Under a stereomicroscope
1 mm
Experiment 4
24 h Unfed
Japanese medaka
Zebrafish
Indian medaka
Clown anemonefish
4 h Fed
green
Excretion time
1, 2, 4, 8, 16, 24 h
count
Counting
Under a stereomicroscope
1 mm


(a) Japanese medaka
(b) Zebrafish
(c) Indian medaka
(d) Clown anemonefish
Particle/fish
0
10
20
30
40
50
60
70
80
90
100
200
300
400
500
600
700
800
Red
Blue
Yellow
Green
Gray
MP color


(a) Japanese medaka
🟥Red 🟦Blue 🟨Yellow 🟩Green ⬜ Gray
12
10
8
6
4
2
0
1
2
3
4
(b) Zebrafish
MP particles
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
(C) Indian medaka
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
Fish ID


(a)
Particles/fish
700
600
500
400
300
200
100
0
*
Light condition
Dark condition
(b)
MP particles
Light condition
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
Dark condition
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
Fish ID
(c)
MP particles
Light condition
450
400
350
300
250
200
150
100
50
0
*
*
Red
Blue
Yellow
Green
Gray
Dark condition
35
30
25
20
15
10
5
0
Red
Blue
Yellow
Green
Gray
MP color


Amount of MP remaining (particles / fish)
Excretion rate of MP (%)
Sampling time (h)
Sampling time (h)
Sampling time (h)
Sampling time (h)
0
1
2
4
8
16
24
0
20
40
60
80
100
0 0 0 0 0
0 0 0 0 0 0
0 0
0 0
0
0
0
0


Amount of MP remaining (particles / fish)
Excretion rate of MP (%)
Sampling time (h)
Sampling time (h)
Sampling time (h)
Sampling time (h)
0
1
2
4
8
16
24
0
0
0
0
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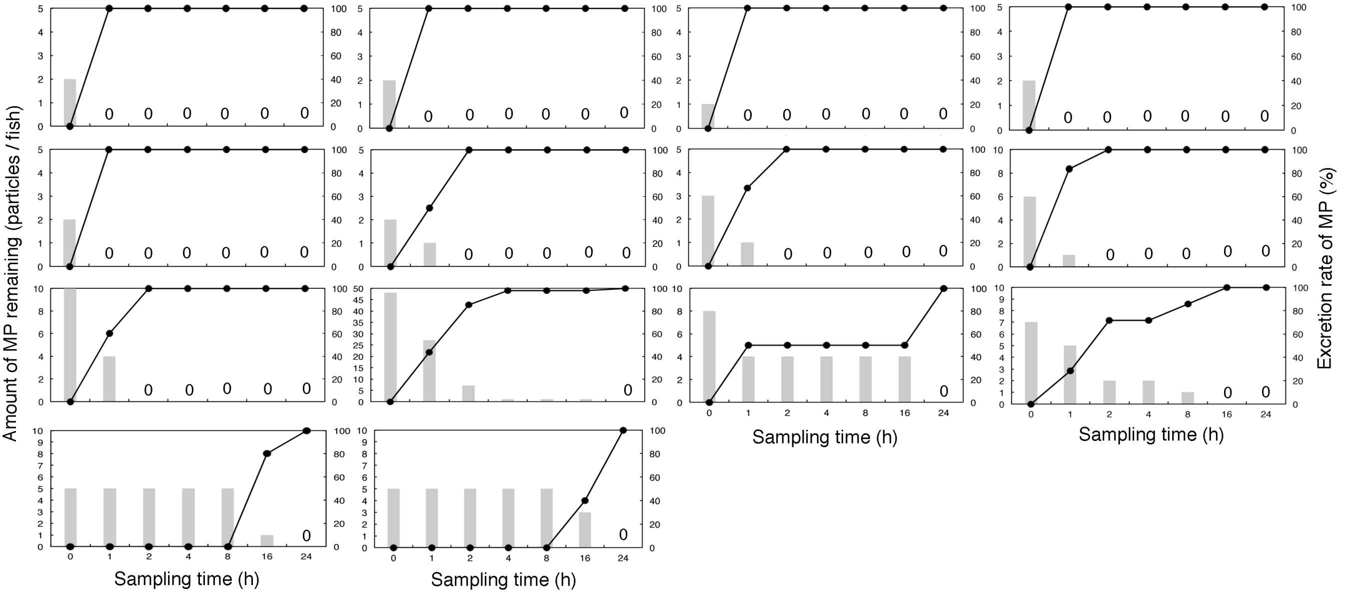
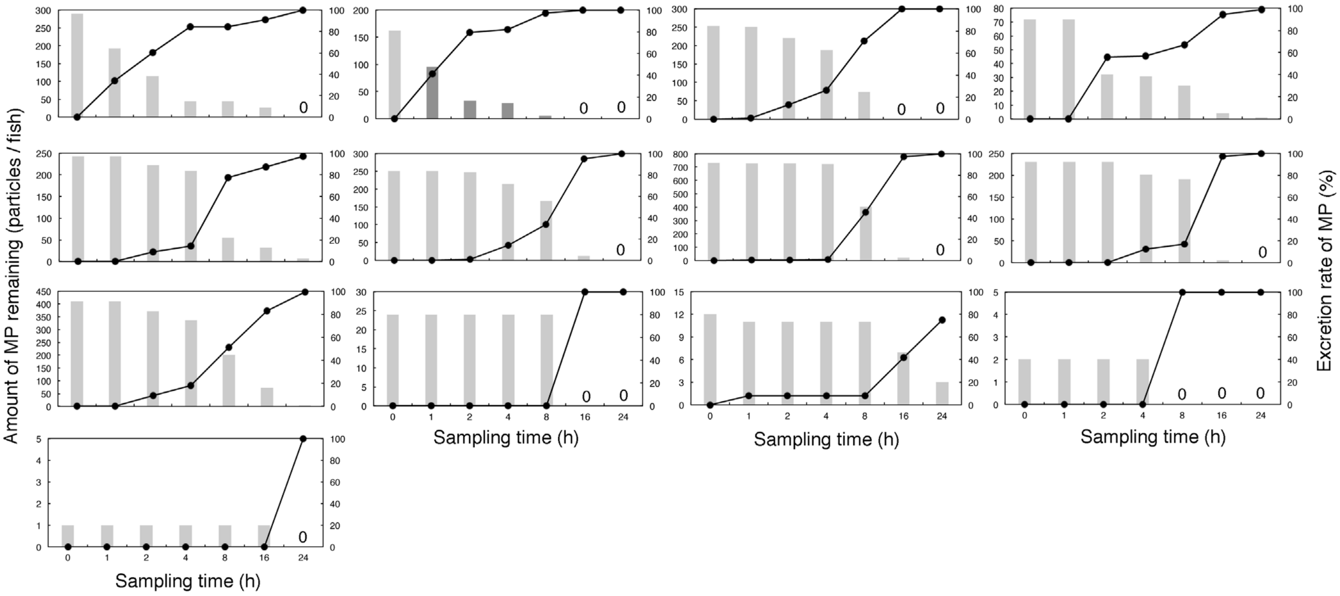
0

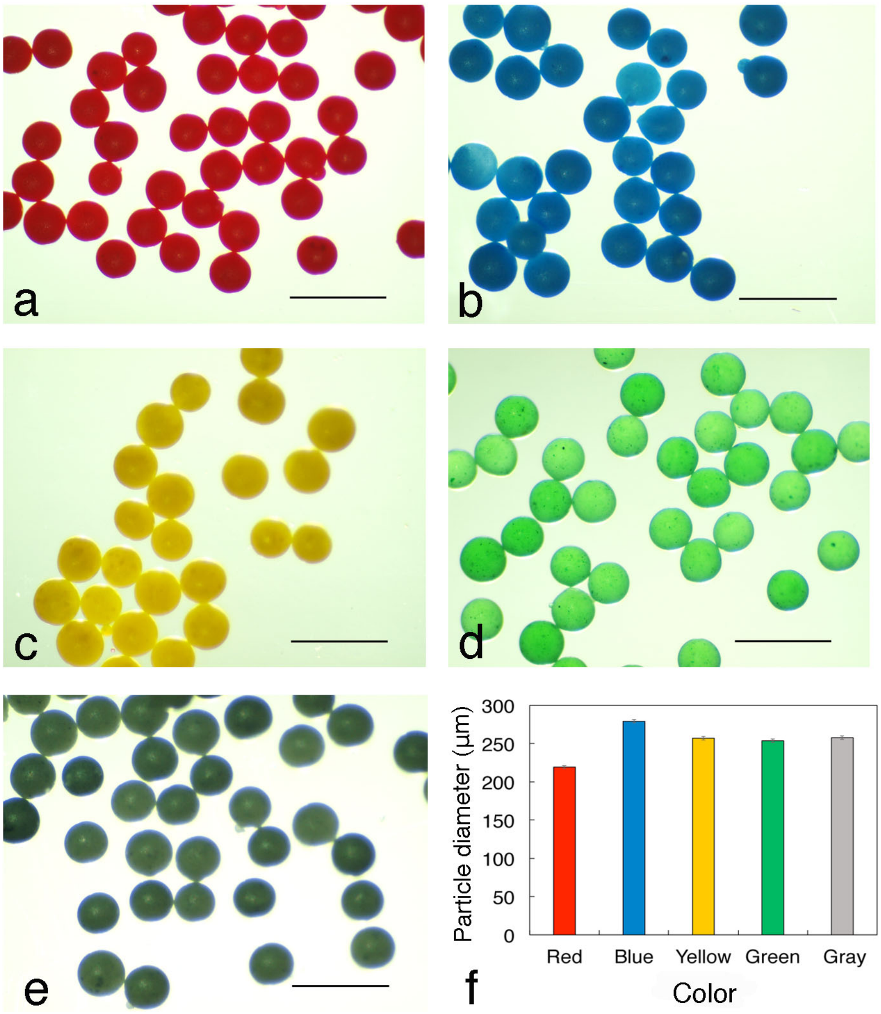

Amount of MP remaining (particles / fish)
Excretion rate of MP (%)
Sampling time (h)
Sampling time (h)
Sampling time (h)
Sampling time (h)
0 1 2 4 8 16 24
0 1 2 3 4 5
0 2 4 6 8 10
0 5 10 15 20 25 30 35 40 45 50
0 20 40 60 80 100
0 0 0 0 0 0


Amount of MP remaining (particles / fish)
Excretion rate of MP (%)
Sampling time (h)
Sampling time (h)
Sampling time (h)
Sampling time (h)
Sampling time (h)
0
1
2
4
8
16
24
0
300
250
200
150
100
50
0
100
80
60
40
20
0
200
150
100
50
0
800
700
600
500
400
450
350
30
25
20
15
10
5
12
9
6
3
70
60
50
40
30
20
10
5
4
3
2
1
0

Table

Table 1. The number of fish that ingested microplastic (MP) of various colors for the species Japanese medaka, zebrafish, Indian medaka, and clown anemonefish.

Species	MP color	Number of fish (percent of total)
Japanese medaka	Red	0 of 14 (0%)
	Blue	3 of 14 (21%)
	Yellow	0 of 14 (0%)
	Green	9 of 14 (64%)
	Gray	3 of 14 (21%)
Zebrafish	Red	12 of 14 (85%)
	Blue	12 of 14 (85%)
	Yellow	12 of 14 (85%)
	Green	12 of 14 (85%)
	Gray	13 of 14 (92%)
Indian medaka	Red	3 of 14 (21%)
	Blue	0 of 14 (0%)
	Yellow	1 of 14 (7%)
	Green	5 of 14 (35%)
	Gray	0 of 14 (0%)
Clown anemonefish	Red	13 of 14 (92%)
	Blue	12 of 14 (85%)
	Yellow	13 of 14 (92%)
	Green	14 of 14 (100%)
	Gray	10 of 14 (71%)


a
b
c
d
e
f
Particle diameter (µm)
300
250
200
150
100
50
0
Red
Blue
Yellow
Green
Gray
Color

Study conception and design: Yoshifumi Horie; data collection: Konori Okamoto; analysis and interpretation of results: Miho Nomura and Hideo Okamura; draft manuscript preparation: Yoshifumi Horie and Konori Okamoto. All authors reviewed the results and approved the final version of the manuscript.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: